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CHROMOSOME INHERITANCE AND REPRODUCTIVE BARRIERS IN BACKCROSSES BETWEEN TWO HYBRIDIZING *VIVIPARUS* SNAIL SPECIES

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ABSTRACT

Hybridization must be followed by repeated backcrossing of the subsequent hybrid generations to the parental species for gene exchange between species to occur. Due to meiotic failures, first-generation hybrids of some species produce unreduced gametes. Their progeny in backcrosses with a diploid parental species are polyploid and functionally sterile. Polyploidy of the backcross generation may therefore act as an instantaneous barrier to gene flow between hybrids and the parental species. Here we determined chromosome inheritance in backcrosses of two hybridizing freshwater caenogastropod snail species to assess whether gene introgression is inhibited in the first backcross generation. *Viviparus ater* and *V. contectus* intermate in nature and produce viable F1 hybrid progeny, although offspring sex ratio is strongly male biased. Despite the different chromosome numbers of the two parental species (*V. ater*, $2n = 18$; *V. contectus*, $2n = 14$), the F1 hybrids are able to reproduce. Allozyme data from natural populations are compatible with gene exchange between the two species, although there is also evidence suggesting that some alleles may be shared because of common ancestry. Our study revealed that all viable backcross progeny were homoploid as they inherited between seven and nine chromosomes from the hybrid father. The siring success of the karyotypically different hybrid sperm was skewed against one sperm karyotype depending on the non-hybrid mother in the cross. In backcross broods of *V. ater* females, the observed distribution of the karyotypes conformed with an assumption of random segregation of two unpaired chromosomes at meiosis in hybrid males. In contrast, when backcrossing hybrid males to *V. contectus* females, post-copulatory processes ultimately determined the karyotype distribution of the backcross progeny. Homoploidy of all backcross progeny together with the presence of sperm and embryos in their gonads makes gene exchange between the two parental species through hybridization possible.

INTRODUCTION

Interspecific hybridization is a common phenomenon in many animal groups, including molluscs, insects, birds, fishes and mammals (e.g. Searle, 1993; Bierne *et al.*, 2006; Mavarez *et al.*, 2006; McCarthy, 2006; Janko *et al.*, 2007). If hybridization leads to gene exchange between interbreeding species, the genetic structure of the original species can be altered, as shown, for example, in pupfishes (Echelle & Connor, 1989). Two conditions must be fulfilled so that gene flow through hybridization can occur: hybrids of the first generation have to be fertile, and hybridization must be followed by repeated backcrossing of subsequent generations to the parental species (introgressive hybridization: Anderson & Hubricht, 1938; Stebbins, 1959). If these two requirements are satisfied and gene introgression occurs, the outcome of a particular hybridizing event depends largely on the extent of gene flow between the parental species mediated by hybrids. In the extreme, extensive unlimited gene flow may lead to the fusion of the gene pools of formerly distinct species and one or both hybridizing species may disappear locally (reviewed by Rhymer & Simberloff, 1996; Gilman & Behm, 2011). This has for instance been assumed to occur in hybridizing benthic and limnetic threespine sticklebacks of the *Gasterosteus aculeatus* species complex (Taylor *et al.*, 2006; Behm, Ives & Boughman, 2010). In contrast, if gene flow is limited, introgression can be a constructive force in evolution (Rhymer & Simberloff, 1996) as it may increase genetic variation in natural populations (Lewontin & Birch, 1966; reviewed by Arnold, 1997).

Reproductive barriers reducing gene flow between hybrids and their parental species are therefore of special interest, not only from an evolutionary perspective, but also in conservation biology.

When two species hybridize and backcross progeny occur, reproductive barriers may inhibit subsequent crossing of the backcross generation with the parental species. In first-generation hybrids, gametogenesis is often hampered because the chromosomes of the two parental species fail to pair properly during meiosis. This can be due to differences in chromosome number or structure, as well as to genetic incompatibilities between the genomes (Maynard Smith, 1985). As a consequence of meiotic irregularities, first-generation hybrids of some hybridizing species generate unreduced diploid gametes (Dannewitz & Jansson, 1996; Garcia-Vazquez *et al*., 2004; Castillo *et al*., 2007). When hybrids producing unreduced gametes mate with diploids, the offspring are allotriploid (e.g. Dannewitz & Jansson, 1996). Allotriploid backcross progeny are not uncommon in fishes (e.g. salmonids: Dannewitz & Jansson, 1996; Garcia-Vazquez *et al*., 2004; Castillo *et al*., 2007). Triploidy can greatly increase the viability of the backcross offspring (Wilkins, Courtney & Curatolo, 1993), but typically their gametes are functionally sterile because they contain unbalanced chromosome complements (Galbreath & Thorgaard, 1995; Mallet, 2007; but see Castillo *et al*., 2007). Subsequent backcrossing of later-generation hybrids with the parental species is one prerequisite for introgression to occur (Anderson & Hubricht, 1938). The karyotype of the first backcross generation must therefore be taken into consideration in order to assess whether gene flow via introgressive hybridization may occur or not.

Backcross progeny with the same level of ploidy as the parental species (i.e. homoploid backcross progeny) may arise when first-generation hybrids produce reduced gametes. Reduced gametes of hybrids between species with different chromosome numbers often have variable chromosome contents (Zong & Fan, 1989), because chromosomes unpaired during meiosis pass more or less randomly to one or other daughter cell (Maynard Smith, 1985). Many of the gametes produced by hybrids may therefore be aneuploid, with some chromosomes occurring twice and other chromosomes wholly absent. Zygotes to which aneuploid gametes have contributed usually die in an early stage of development (Maynard Smith, 1985). If fertile balanced gametes capable of siring offspring occur in hybrids between species with different chromosome numbers (e.g. in mules and hinnies: Trujillo *et al*., 1969; Chandley *et al*., 1974, Rong *et al*., 1988; Zong & Fan, 1989), it is generally not possible to predict accurately the karyotype of the backcross progeny for two reasons. First, balanced gametes may arise due to several meiotic configurations (Yang *et al*., 2004). Secondly, post-copulatory selection may influence the siring success of sperm with different karyoypes (Chayko & Martin-DeLeon, 1992; Dernburg *et al*., 1996). Post-copulatory selection based on sperm karyotype has, for example, been reported in two chromosomal races of the alpine grasshopper *Podisma pedestris*. After mating with males of both chromosomal races, females were preferentially fertilized by males of their own karyotype (homogamy: Hewitt, Mason & Nichols, 1989). This suggests that selection acting after sperm transfer favours the most compatible sperm, which is most likely the sperm with a karyotype most similar to that of the female.

In this study, we determined the karyotype of the backcross generation of hybrids between two freshwater caenogastropod snails to test if introgression is inhibited in later backcross generations. The original species *Viviparus ater* (Cristofori & Jan, 1832) and *V. contectus* (Millet, 1813) are dioecious and hybridize in nature (Trüb, 1990; Ribi & Oertli, 2000), despite having different chromosome numbers (*V. ater*: $2n = 18$; *V. contectus*: $2n = 14$). All chromosomes of both species are metra- or submetracentric, and similar in size and shape (Barsiene, Ribi & Barsyte, 2000). Allozyme data are in agreement with the hypothesis of gene exchange between the two species (Porter & Ribi, 1994), but there is also evidence suggesting that alleles may be shared because of common ancestry (Katoh & Ribi, 1997). First-generation hybrids are predominantly males (Trüb & Ribi, 1997) and have 16 chromosomes (Sbilordo, 2010). Although meiosis in hybrid males is irregular and multivalent associations and univalents were found in almost all meiotic cells, males produce numerous sperm of normal appearance (Sbilordo, 2010) and are fertile in interbreeding experiments in backcrosses with both parental species (Trüb & Ribi, 1997). The existence of fertile hybrid males in these internally fertilizing animals provides an excellent opportunity to study the role of the different chromosome sets of the parental species in producing reproductive barriers beyond the initial hybrid generation.

Based on the karyotype of the backcross generation, we first evaluated whether first-generation hybrids produced reduced or unreduced gametes. This would allow verification of the first necessary condition for introgression to occur: i.e. whether backcrossing of later-generation hybrids to the parental species is even possible. The production of unreduced gametes may be restricted to one parental combination of hybrids, as has been shown in salmonids. Salmonid hybrids arising from a cross between a male trout (*Salmo trutta*) and a female salmon (*Salmo salar*) generated unreduced gametes, while hybrids of the reciprocal parental combination did not (Garcia-Vazquez *et al*., 2004). Therefore, secondly, we asked whether the parental combination of the hybrid males affects the chromosome contents of their sperm. Finally, we asked whether the pure species mother influences the number of chromosomes inherited from the hybrid father in backcrosses. This would indicate that post-copulatory processes influence the karyotype of the backcross generation.

MATERIAL AND METHODS

Breeding of F1 hybrids

Viviparus pairs consisting of a virgin female and a heterospecific male were set up in underwater cages in Lake Zürich in June 2002 (for further details of the underwater installation, see Trüb, 1990; Trüb & Ribi, 1997). The ancestors of all *Viviparus contectus* originated from Lazise, Lake Garda, Italy. The ancestors of the *V. ater* females originated either from Lazise or from Lake Zürich, the *V. ater* males were collected at Küsnacht, Lake Zürich.

In September, the males in the cages were replaced to reduce the risk of fecundity loss by infection with castrating larval trematodes (Oppliger, Hosken & Ribi, 1998; Sbilordo, 2001) and snails were then left to hibernate. From April to September of the following year, hybrid offspring were removed monthly by scuba diving and housed separated by cross type (*V. ater* male × *V. contectus* female; *V. contectus* female × *V. ater* male). During these dives, the cages were cleaned of algae, and damaged or overgrown mesh cones were replaced when necessary. Although *Viviparus* snails feed on detritus (Ribi and Arter, 1986) and are able to filter particles suspended in the water column (Cook, 1949), they were fed with cooked carrots to enrich their diet. After the second hibernation period at the end of May 2004, all snails were removed and kept in an air-conditioned room at 18°C for about 1 week until the hybrids were used in the backcross experiment. During this period, the snails were fed with plant chips (Vitakraft).

Breeding of backcrosses

Backcrosses between F1 hybrid males and females of each of the two parental species were performed in the same cages as the F1 crosses (see above). The backcross pairs, consisting of a hybrid male and a virgin female of one of the parental species, were set up between late May and early June 2004. The cages were censused five times between June and the end of September. At the beginning of September, the first backcross offspring were born. Offspring were removed at the end of September and housed indoors in ventilated plastic shelters separated by families until they were old enough to be sexed and the males ($n = 10$) were used for karyological analysis. In April 2006, the breeding was terminated and additional backcross offspring were assessed. Only large males ($n = 47$) with at least one clearly visible annual ring on the shell were used from this second sample (see Mutzner, 1986) to avoid any risk of analysing later-generation offspring. Since adding the data from the two samples did not influence the outcome of the study, we pooled the data.

Slide preparation

Metaphase spreads were prepared from gonadal cells of the male backcross progeny. Dissected gonads were cut into small pieces and treated in an aqueous colchicine solution (0.125%) for 90 min at room temperature to arrest cell division in the mitotic stage. The colchicine solution was renewed after 60 min. The hypotonic treatment was subsequently finished in bidistilled water for 30 min. During the hypotonic treatment, the bidistilled water was changed three times. The tissue was then fixed in freshly prepared ice-cold 1:3 acetic acid:ethanol for 16 h. During fixation, the fixative was changed after 30 min, after 60 min, after 6 h and immediately before slide preparation. New microscope slides were cleaned in 10% Deconex and then stored in 70% ethanol at 4°C until use. Small pieces of fixed tissue were crushed in a 60% acetic acid solution and three drops of the cell suspension were placed on a slide. When the surface of the cell suspension became grainy, the slide was passed upside down through water vapour for 3 s and then dried on a heated plate at 60–65°C (Henegariu *et al*., 2001). After washing, the slides were left to cool down at room temperature, then stained in a 4% Giemsa phosphate buffer (pH = 7), rinsed with tap water and mounted in Euparal (Chroma, Stuttgart).

Table 1. The number of the backcross offspring analysed per family

Father	Mother	Number of families	Offspring analysed	Offspring per family
axc	a	5	15	6,4,3,1,1.
axc	c	4	15	7,3,3,2.
cxa	a	4	12	5,4,2,1.
cxa	c	5	15	3,3,3,3,3.

a: *V. ater*; c: *V. contectus*; axc: hybrid, father *V. ater* and mother *V. contectus*; cxa: hybrid, father *V. contectus* and mother *V. ater*.

Sample size and analysis

The aim of the interbreeding was to obtain 15 backcross males for karyological analysis of each of the four possible crosses between a hybrid male and a female of the parental species. However, from one backcross combination, only 12 males were obtained for karyotype assessment (Table 1). The modal chromosome numbers of backcross offspring were assessed by counting the chromosomes in at least 20 mitotic cells per male, apart from one exceptional case where the low mitotic activity only allowed counting the chromosomes of 10 cells. Cells with modal chromosome sets were observed in 82% of the studied cells overall or in 76.8–85% of the cells when looking at the four backcross combinations separately (Table 2). In *Viviparus* snails, some variability in chromosome counts is natural (see Barsiene *et al*., 2000) and there was no significant difference in the frequency of cells with aberrant chromosome sets between the progeny of the four backcross combinations, which would indicate a discrepancy in postzygotic mitotic failures (GLM: $F_{3,53} = 2.427$, $P = 0.076$; Kolmogorov–Smirnov normality test on residuals: $P = 0.483$).

For statistical analyses, SPSS v. 20.0 was used. To test the effects of the pure mother and the parental combination of the hybrid father on the chromosomes inherited by the backcross progeny, we used two separate Mann–Whitney *U* tests. To account for multiple testing, we applied the Bonferroni correction of the *P* values. The relationship between the numbers of chromosomes inherited from the hybrid mother in backcrosses was analysed with a Spearman correlation. Finally, a χ^2-test was used to examine whether the observed pattern of chromosome inheritance in the backcross progeny could by explained by the random segregation of two unpaired chromosomes during meiosis in hybrid males.

RESULTS

The diploid chromosome complement of F1 hybrid males of both reciprocal crosses consists of 16 chromosomes, 7 originating from *Viviparus contectus* and 9 from *V. ater* (Sbilordo, 2010). When backcrossing these F1 hybrid males to females of both parental species, all resulting male offspring were homoploid, independent of the parental combination of the hybrid father. The offspring of *V. ater* females generally had larger chromosome numbers than those of *V. contectus* females. Specifically, *V. ater* females gave birth to backcross offspring with 16–18 chromosomes whereby the majority of them (67%) had 17 chromosomes. In contrast, the backcross progeny of *V. contectus*

Table 2. Chromosome number variability in the backcross progeny

Father	Mother	No. of snails	No. of cells studied	Cells with modal chromosomes (%)	Cells with less than modal chromosomes (%)	Cells with more than modal chromosomes (%)
axc	a	15	328	76.8	13.7	9.5
cxa	a	12	230	82.6	12.6	4.8
axc	c	15	313	84.0	9.6	6.4
cxa	c	15	300	85.0	6.3	8.7
total backcrosses		57	1171	82.0	10.5	7.5

a: *V. ater*; c: *V. contectus*; axc: hybrid, father *V. ater* and mother *V. contectus*; cxa: hybrid, father *V. contectus* and mother *V. ater*.

females contained either 15 (40%) or 16 (60%) chromosomes. The higher chromosome numbers in backcross progeny of *V. ater* mothers is due to the greater number of chromosomes in the maternal species (18 *vs* 14 chromosomes: Rainer, 1963; Barsiene *et al*., 2000).

Thirteen out of the 15 parental pairs from which more than one offspring was analysed produced backcross progeny with different chromosome numbers. This result indicates that the sperm of an individual hybrid male was not identical in their chromosomal contents. As the mother was always a female of one of the parental species, the numbers of chromosomes the backcross offspring inherited from the hybrid father can be inferred based on the karyotypes of the mother. Fertile hybrid sperm contained between seven and nine chromosomes (Fig. 1). Interestingly, the species of the non-hybrid mother significantly influenced the chromosome contribution of the hybrid father to the backcross brood (Mann–Whitney *U*-test: $z = -3.456$, $P = 0.001$) and there was a significant negative correlation between the maternal chromosome number and the number of chromosomes inherited from the hybrid father (Spearman correlation coefficient $= -0.462$, $P < 0.001$). When hybrid males were backcrossed to *V. ater* females ($2n = 18$), most of the offspring contained eight chromosomes of hybrid origin and offspring with seven or nine paternally inherited chromosomes occurred at lower frequencies (Fig. 1). This pattern of chromosome inheritance does not deviate significantly from the expectation of a binomial distribution assuming random segregation of two unpaired chromosomes at meiosis in hybrid males (χ^2-test: $\chi^2 = 3.074$, df $= 2$, $P = 0.215$). In contrast, in backcross broods of *V. contectus* females ($2n = 14$), most of the progeny contained nine chromosomes of hybrid origin and offspring with seven paternally inherited chromosomes were never found (Fig. 1). Thus, random segregation of two chromosomes in hybrid males cannot explain the pattern of chromosome inheritance observed in the backcross progeny (χ^2-test: $\chi^2 = 22.80$ df $= 2$, $P < 0.001$).

The parental combination of the hybrid father had no statistically significant effect on the number of chromosomes passed from the hybrid father to the backcross progeny (Mann–Whitney *U*-test; $z = -1.242$, $P = 0.214$). Images of mitotic chromosome spreads of first-generation hybrid males and some backcross progeny are given in the Supplementary material.

DISCUSSION

The main finding of our study is that the backcross progeny of all crosses analysed were homoploid, independent of the parental combination of the hybrid father. This makes introgression of genes into the parental species through further backcrossing a real possibility. The hybrid males produced fertile sperm with variable chromosome numbers. When hybrid males were backcrossed to females of the parental species, fertilization success of the karyotypically different hybrid sperm was dependent on the female in the cross. The karyotype distribution found in backcross broods of *V. ater* females was consistent with an expectation of random segregation of two unpaired chromosomes at meiosis in hybrid males. In contrast, the karyotypes observed in backcrosses with *Viviparus contectus* females indicated that postcopulatory processes were responsible for the karyotype distribution observed in the backcross generation. Postcopulatory selection can act either between insemination and fertilization, or later on when zygotes are formed (e.g. Howard *et al*., 2009; Immler *et al*., 2011). Both prezygotic selection on sperm and postzygotic offspring inviability may have contributed to the observed karyotype distribution in the backcross progeny of *V. contectus* females.

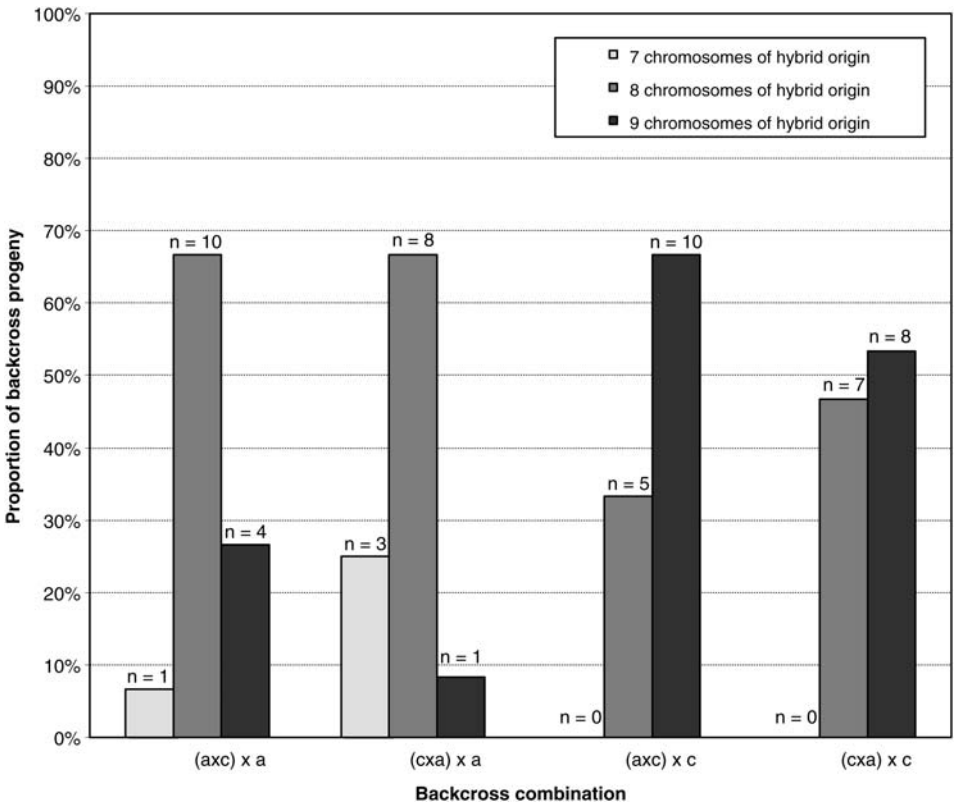

Figure 1. Hybridization in *Viviparus*: the number of chromosomes inherited by the backcross progeny from the hybrid father in the four possible backcross combinations with hybrid males (n = number of offspring). axc: hybrid male, father *V. ater* and mother *V. contectus*; cxa: hybrid male, father *V. contectus* and mother *V. ater*; a: *V. ater* female; c: *V. contectus* female.

Postcopulatory prezygotic selection on sperm may result from sperm competition (Parker, 1970), whereby sperm better adapted to the environmental conditions in the female reproductive tract are superior in fertilization (reviewed in e.g. Eberhard, 1996; Birkhead & Møller, 1998; Simmons, 2001; Pitnick, Wolfner & Suarez, 2009; Pizzari & Parker, 2009.). Alternatively, females may actively select the most compatible sperm to fertilize their eggs, or bias against less compatible sperm through inhibition (cryptic female choice: Eberhard, 1996; Zeh & Zeh, 1997). The processes involved in sperm competition and cryptic female choice generally refer to sperm from more than one ejaculate. However, we expect these processes also to operate within a single ejaculate when karyotypically variable sperm occurs. Evidence for prezygotic selection of sperm within an ejaculate based on sperm karyotypes has been reported by Chayko & Martin-DeLeon (1992). By comparing the fertilization success of chromosomally normal sperm of the mouse with those of translocation-X-bearing sperm in vivo and in vitro, they demonstrated that the female-imposed conditions in which fertilization occurs significantly affect the fertilization success of karyotypically different sperm within an ejaculate.

The 'genetic compatibility hypothesis' predicts that sperm superior in fertilization are those most compatible with the genome of the female or those that at least minimize genetic incompatibility (reviewed by Zeh & Zeh, 1997; Howard *et al*., 2009; Pitnick *et al*., 2009). Therefore, in hybrid crosses, conspecific sperm generally have a competitive advantage over heterospecific or hybrid sperm (conspecific sperm precedence: e.g. Hewitt *et al*., 1989; Price, 1997; Howard *et al*., 1998; Immler *et al*., 2011). In contrast, our results do not provide support for conspecific sperm precedence when looking at the chromosome numbers of the hybrid sperm successful in fertilization. While sperm with hybrid-like eight chromosomes fathered most of the offspring in backcrosses with *V. ater* females, sperm with *ater*-like 9 chromosomes were most successful in backcrosses with *V. contectus* females. These findings seem to indicate a pattern of heterospecific sperm precedence, although we do not know exactly how the chromosomes from the species of origin contributed to the different hybrid sperm.

The high fertilization success of sperm with eight chromosomes generally observed in the backcrosses may be a consequence of a quantitative predominance of this sperm type in the hybrid ejaculates. Assuming that two *V. ater* chromosomes lack a matching counterpart at meiosis in hybrid males, and that these two chromosomes are therefore passed randomly to one of the daughter cells, half of the produced sperm should contain eight chromosomes. In contrast, the prevalence of offspring fathered by sperm with nine chromosomes in backcrosses with *V. contectus* females cannot be explained without invoking some form of selection mechanism.

The few studies reporting a higher fertilization success of heterospecific sperm or sperm from a more distantly related species include, for example, starfish (Harper & Hart 2005) and backcross females of hybrids between the two cricket species *Allonemobius fasciatus* and *A. socius* (Britch *et al*., 2007). Heterospecific sperm precedence may result from sexual conflict. Through sexually antagonistic coevolution, females may have evolved strong resistance to conspecific but not to heterospecific sperm, leading to increased fertilization success of heterospecific sperm after interspecific matings (Parker & Partridge, 1998; Martin & Hosken, 2004; Arnqvist & Rowe, 2005; Mendelson, Imhoff & Venditti, 2007). Although the mechanism for the observed sperm precedence in backcrosses with *V. contectus* females is not yet known, it may be that female-mediated processes are involved. *Viviparus contectus* females are able to ingest sperm in the epithelial cells of the reproductive tract after insemination (Dembski, 1968). This makes sperm choice by females through digestion of particular sperm types before fertilization plausible.

An alternative explanation for the observed karyotypes of the backcross offspring may be that zygotes or embryos with less than 15 chromosomes are simply unable to survive. In support of this notion, in backcrosses with a *V. contectus* female where the ova contain seven chromosomes, none of the backcross progeny had less than eight chromosomes of hybrid origin. In contrast, when hybrids were backcrossed to *V. ater* females with ova containing nine chromosomes, sperm types with 7–9 chromosomes were fertile. In animals, chromosome deficiencies often have lethal effects. For example the lack of two chromosomes in the hybrids of the two flatfish species *Paralichthys dentatus* and *P. olivaceus* causes morphological deformation and death at an early development stage (Xu *et al*., 2009; Sui, Liu & He, 2011). Consistent with the argument that offspring of *Viviparus* hybrids with less than 15 chromosomes may not be viable, Trüb (1990) occasionally found aborted embryos in backcrosses with *V. contectus* females in his interbreeding experiments. Nevertheless, it seems unlikely that embryo mortality alone could have caused the observed bias in fertilization success of hybrid sperm in backcrosses with *V. contectus* females, because one of the backcross combinations between a hybrid male and a *V. contectus* female was in fact even more fecund than intraspecific *V. contectus* control crosses (see Trüb & Ribi, 1997).

In conclusion, in sympatric populations of *V. ater* and *V. contectus*, the conditions for gene introgression through hybridization seem to be fulfilled (see Anderson & Hubricht, 1938). Previous work showed that first-generation hybrids occur in natural populations (Katoh & Ribi, 1996) and that these hybrids are fertile in backcrosses with both parental species in non-competitive experiments (Trüb, 1990; Trüb & Ribi, 1997). Here we further show that the resulting backcross offspring are homoploid recombinants, as more than 92% had intermediate chromosome numbers (i.e. 15–17 chromosomes). Sperm production observed in backcross males and eggs found in backcross females indicate that backcross progeny are able to reproduce (S. Sbilordo, personal observation). Thus subsequent crossing of the backcross generation with the parental species seems plausible. However, the fertilization success of heterospecific and hybrid sperm should be tested in competition against conspecific sperm to assess the effect of hybridization on the integrity of the parental species (see e.g. Arnold, 1997; Howard *et al*., 2009). *Viviparus* females are promiscuous and mate frequently (Staub and Ribi, 1995), and *V. contectus* females have been shown to be able to store sperm for more than 2 years (Trüb, 1990). Hybrids and potential backcross hybrids only occur at very low frequencies of less than 1.7% in natural populations (Katoh & Ribi, 1996). Therefore, most females mating with a heterospecific or a hybrid male are likely to have already stored conspecific sperm from a previous mate or to mate soon afterwards with a male of their own species. Although sperm with a heterospecifc or hybrid-like chromosome number were superior in fertilization within a hybrid ejaculate, conspecific sperm precedence may occur when hybrid sperm are competing for fertilization with sperm from a conspecific male (e.g. Howard *et al*., 2009; Immler *et al*., 2011). Therefore, investigation of sperm selection mechanisms operating after insemination by a conspecific and a heterospecific or a hybrid male would be required to determine the actual influence of hybridization and introgression on the genetic architecture of the parental species.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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